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PRE-TREATMENT AND ENZYMATIC HYDROLYSIS OF BANANA (*Musa acuminata x balbisiana*) PSEUDOSTEM FOR ETHANOL PRODUCTION

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Abstract. Banana (*M. acuminata x balbisiana*) is an abundant lignocellulosic waste material in large plantations all over the Philippines, especially in Mindanao, which can be utilized as substrate in producing high-value products like ethanol. To compensate for the low yield based on total weight of substrate due to the high moisture content of banana pseudostem, there is the primary challenge to make the conversion of this lignocellulosic biomass into monomeric sugar and then into ethanol more efficiently in order to achieve yields that would make it cost-competitive. Hence, this study evaluated the effects of solid loading, incubation time and amount of enzyme on yield of reducing sugars in the enzymatic hydrolysis process and attempted to optimize the significant factors by Response Surface Methodology (RSM), specifically using Box-Behnken design. There was significant improvement on the reducing sugar yield of the pretreated banana pseudostem at 20 h incubation time, 15 g solid loading and 0.55 % enzyme concentration. Ethanol production was observed to be higher in the detoxified substrate although biomass was higher for the non-detoxified substrate. As to our knowledge, the present study is the first attempt to produce second generation ethanol using banana pseudostem waste as feedstock in the Philippines.
Keywords: ethanol fermentation, banana pseudostem, Response Surface Methodology, Box-Behnken Design

INTRODUCTION

Diminishing sources of fossil fuels had increased worldwide interests in finding alternatives to energy resource in a more sustainable fashion (Reddy et al., 2010) including lignocellulosic agricultural waste materials or biomass (Sun & Cheng, 2002) and food wastes (Girotto et al., 2015) for biofuel production, particularly referred to as second generation bioethanol. In the Philippines, banana is one of the most important fruit crops in terms of production volume and export earnings. Banana farming produces banana plant wastes, particularly the leaves and pseudostem which are bulky and fibrous and cannot be broken down easily through natural process of decomposition (Calderon & Rola, 2003). These residual biomass from banana are amilaceous and lignocellulosic which should be initially hydrolyzed into glucose thru chemical or enzymatic hydrolysis before being used as feedstock for ethanol fermentation (Arredondo et al., 2009).

In the study conducted by Filho et al. (2013), pseudostem from *M. cavendischii* banana tree was used as a substrate for alcoholic fermentation. They were able to get satisfactory results with the maximum yield of ethanol formed per unit of substrate consumed, total productivity and conversion efficiency values of 0.35g, 0.90g ethanol $L^{-1} \cdot h^{-1}$ and 65.9%, respectively. In another study by Ingale et al. (2014), two fungal strains, *Aspergillus ellipticus* and *A. fumigatus*, were used as saccharification pre-treatment agents to facilitate maximum release of reducing sugars from *M. acuminata* pseudostem. The

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hydrolysate obtained after treatment was fermented by *Saccharomyces cerevisiae* NCIM 3570 to produce ethanol. Fermentation of cellulosic hydrolysate gave maximum ethanol of 17.1 gL^{-1} (84% yield) and productivity of 0.024 $g\%h^{-1}$ after 72 h. From the study of Souza et al. (2014), they were able to find out that fresh biomass from banana pseudostem pre-treated with NaOH had greatest percent yield in reducing sugar (YRS = 79.5±4.4 %), wherein it was 84% higher compared to pre-treated dry biomass with the same hydrolysis catalysis, and 31% higher than the value reached in the pretreatment of the same biomass with H_2SO_4. But maximum reducing sugar (RS) value in hydrolyzed liquor was obtained from dry biomass saccharification with H_2SO_4 with a value of 26.6±1.1 gL^{-1}. Fermentation of this liquor, after concentrating to RS ≤ 62.1 gL^{-1}, showed ethanol production of 22.1±0.8 gL^{-1} with respective values of YP/RS = 0.47±0.03 g/g, ethanol productivity (QP) 1.83±0.12 gL^{-1}·h and conversion efficiency of 80.4±0.12 %.

In this study, the potential use of *M. acuminata x balbisiana* pseudostem as raw material for second-generation ethanol production was also investigated. Specifically, it aimed to optimize and determine the effects of different solid loading, incubation time and amount of enzyme on yield of reducing sugars (YRS) in the enzymatic hydrolysis process of banana pseudostem. Better ethanol yield but with low production cost need optimization of process. Optimization of one factor at a time is simple, but this one is time consuming and often fails to seek the optimum region because of the joint effects of factors that are not considered. Response Surface Methodology (RSM) is a better option, being used to study aggregate effects of variables and seek optimum conditions for this multivariable system in various industries (Yolmeh and Jafari, 2017). In this study, banana pseudostem hydrolysis was optimized using RSM-based Box-Behnken Design (BBD) with reducing sugars concentration as response variable.

METHODS

Collection and Preparation of Materials

A total of 23.4 kg banana (*M. acuminata x balbisiana*) pseudostem or trunk was collected from the premises of BIOTECH-UPLB, College, Los Baños, Laguna, Philippines and processed immediately. The fresh pseudostem with moisture content (MC) of approximately 90% (Ambrose and Naik, 2016) was cut into small pieces of approximately 0.5" thickness x 2" width x 2" length and was dried in a locally manufactured cabinet type oven dryer of size 72" height x 72" length x 36" width at 80^0C for 72 h. The dried plant material with MC of approximately 6% (d.b.) was then passed through a grinder to obtain 40 mm mesh powdered samples. A total of 1.3 kg banana pseudostem powder or equivalent to 5.6% (d.b.) yield was obtained.

Alkali Pre-treatment

Powdered banana pseudostem was pretreated with 3% NaOH solution. One hundred grams of the pseudostem was added to one liter of 3% NaOH solution and placed in a 2-L Erlenmeyer flask. The mixture was then autoclaved at 121^0C (15 psi) for 15 min. The pretreated samples were then neutralized after inversion method by adding 1NHCl before analysis of the reducing sugar content using Dinitrosalicylic (DNS) method (Miller, 1959).

Optimization of Enzymatic Hydrolysis of Banana Pseudostem

Response Surface Methodology (RSM), a collection of mathematical and statistical techniques that are useful in modelling and analysis of problems in which a response is influenced by several variables (Myers et al., 2003) was used to optimize enzymatic hydrolysis of banana pseudostem. In this study, RSM using a Box Bhenken Design (BBD) model was used to identify the experimental setups. Three conditions, namely, solid loading (g), incubation time (t),

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and enzyme loading (%), were set as variables for the setup as presented in Table 1. The flasks were incubated at 50^0C water bath until the saccharification process was completed. An aliquot of one mL was taken from each setup and was subjected for DNS assay.

Cellulase Assay

Filter paper assay for saccharifying cellulase (FPU Assay) was done according to the method by Ghose (1987) as modified by Adney & Baker (1996). The powdered enzyme was dissolved (1g/100mL) in Na-citrate buffer (1.21 g citric acid, 1.98 g Na-citrate into 500 mL distilled water) at pH 4.8. One mL of Na-citrate buffer was poured in a test tube containing a filter paper (1 cm x 6 cm Whatman; 50 mg) and incubated at 50^0C for 10 minutes. Then, 0.5 mL enzyme of preparation was then added. The setups were incubated at 50°C for 1 h. A reagent blank (1 tube with 1.5 mL citrate buffer only), a substrate control (1 tube with 1.5 mL citrate buffer and filter paper), and glucose standards (8 tubes) were also prepared. The samples, enzyme blanks, glucose standards and the spectro zero were added with 3.0 mL DNS reagent, mixed and boiled together in a water bath for 5 min. Then, tubes were cooled in an ice bath for 10 min. Five (5.0) mL distilled water was added to 0.4 mL of each sample. Tubes were mixed by completely inverting the tube several times. The sample reaction tubes, enzyme blank and standards were measured against the spectro zero at 540 nm. The enzyme blank was used to measure the color against spectro zero and subtracted from the value of the appropriate reaction tube.

Detoxification

Detoxification was done in order to minimize inhibition during yeast fermentation. The saccharified hydrolysate was subjected to detoxification by adding 8.0 g (4% w/v) activated carbon per 200 mL in a flask. Incubation was done at 35^0C with shaking at 100 rpm for 2 h. The mixture was then centrifuged and decanted to remove the activated carbon. The detoxified liquid extract was then stored inside the freezer prior to fermentation. Approximately >1.0 mL sample was obtained for DNS Assay. The samples were stored in the freezer before analysis.

Upscale Saccharification

Upscale saccharification was done using a saccharification reactor (Fig. 1.) located at the Fermentation and Engineering Service Laboratory, BIOTECH, UPLB. A total of 493.5 g pre-treated sample was transferred to 5.0 L Na- citrate buffer (12.08 g citric acid, 19.85 g Na-citrate into 5.0 L distilled water). Based from the optimized flask studies, 18.10 mL of cellulase enzyme was added to the setup before loading into the reactor. The mixture was incubated at 50^0C with 30 rpm rotary mixing for 30 h.

Ethanol Fermentation

S. cerevisiae BIOTECH Strain 2030 was obtained from the culture collection of the Biotechnology for Industry, Energy and Environment Program, BIOTECH-UPLB. The pure culture was streaked into YEPD agar (10 gL^{-1} each of yeast extract and peptone, and 20 gL^{-1} each of dextrose and agar) slants and incubated at 35^0C for 5 days. The cells were then harvested and transferred to 50 mL YEPD broth. The broth was incubated at 35^0C for 24 h with shaking at 100 rpm. The cells were aseptically collected by decanting and optical density at 660 nm was adjusted to 1.0 (corresponding to approximately 1.0 x 10^8 cells mL-1) and then inoculate at a rate of 2% (v/v) or two mL to 100 mL alcohol fermentation medium in flask with bent tube covering equipped with catchment bulb containing H_2SO_4 (Fig. 2). The 2% (v/v) concentration of the yeast inoculum was based on the usual practice in the laboratory (Madigal et al., 2019) but which is less than that mentioned in a previous fermentation study on banana pseudostem by Filho et al., (2013) which was 20% (v/v).

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Figure 1. Saccharification Reactor used in the upscale enzymatic hydrolysis of pretreated banana pseudostem. The major components are reaction tank with rotating screw and jacket (A), motor (B), electrically-heated water drum for temperature control (C) and water pump (D).

The main substrate consisted of banana pseudostem hydrolysate supplemented with 1.4 gL^{-1} $(NH_4)_2SO_4$, 1.0 gL^{-1} KH_2PO_4 and 0.05 gL^{-1} $MgSO_4$ and pH adjusted to 5.0 prior to sterilization (NIBAM-UPLB Training Manual, 1987). Media were sterilized for 15 minutes (15 psi) at 121^0C. For this flask setup, changes in weight were monitored every 2 hours to account for CO_2 evolution. For the bioreactor run, the setup contained 3.0 L of alcohol fermentation medium added with the supplements mentioned above. Inoculation rate was 300 mL of the yeast inoculum grown in YEPD broth. Fermentation was carried out in a 5-L bioreactor for 8 h at ambient condition and slow agitation (50 rpm) (Fig. 3). Ethanol was assayed after the run.

Figure 2. Flask fermentation Set-ups

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Figure 3. Upscale fermentation set-up using BioStat Bioreactor

Reducing Sugar Assay

Reducing sugar was assayed by the DNS (Dinitrosalicylic acid) colorimetric method by Miller (1959). One milliliter was taken from each setup of the substrate stock solution. An aliquot of 0.5 mL sample was added to 1.5 mL DNS reagent. The mixtures were placed in boiling water bath for 15 min. After boiling, the mixtures were cooled down in ice bath and diluted with 10 mL distilled water. The absorbance of each samples were read at 550 nm. Distilled water served as blank for the samples assayed before and after saccharification and fermentation.

Determination of Ethanol Concentration

Gas Chromatography (GC) was performed to evaluate the ethanol content produced after fermentation of the banana pseudostem hydrolysate using Shimadzu model 2014 (Japan) equipped with Flame Ionization Detector and automatic injector. Five (5.0) mL of the sample were obtained and centrifuged at 10,000 rpm for five minutes. The supernatant was collected and filtered through 0.45 uL filter. To prepare the standard ethanol calibration curve, separate aliquots of 0.1, 0.5, 1.0, 3.0, and 5.0 mL absolute ethanol were placed in properly-labeled capped test tubes, diluted with 100 mL distilled water and mixed thoroughly. One milliliter of sample solution or standard ethanol solution and 1.0 mL of 2 % isopropanol and 3.0 mL distilled water were mixed well. Then, 1.0 µL of each sample was injected into the gas chromatograph and the respective peak areas of ethanol and isopropanol were obtained. The peak ratio of ethanol to isopropanol for every standard solution of ethanol was calculated and plotted on the x-axis (ethanol concentration) and the ratio of ethanol peak area to isopropanol peak area on the y-axis. Ethanol values for the samples were obtained from the standard ethanol calibration curve.

RESULTS AND DISCUSSION

Optimization of Saccharification Parameters

Optimal parameters for saccharification of pre-treated banana pseudostem were determined

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by conducting small-scale saccharification in 250-mL flasks with varying incubation time, enzyme concentration and solid loading. The novozyme cellulose used was assayed to have an activity of 0.526 U. RSM, using the Box Bhenken Design, was performed in order to study the combined effects of different variables on saccharification efficiency and to determine the optimum parameters that would yield the highest concentration of reducing sugar (Chittibabu et al., 2012), assayed as glucose through DNS method. ANOVA results suggested that solids loading significantly affected the reducing sugar yield during saccharification (data not shown). The model was also significant whereas there was no significant lack of fit. The results are shown in Table 1. The parameters for run 3 (20 h incubation time, 15.00 w/v solid loading and 0.55 % enzyme concentration) seemed to give the most cost efficient conditions with the second highest reducing sugar concentration of 24.43 gL^{-1}. The surface response three dimensional model graph is shown in Figure 4 showing the optimum values. The parameters which resulted to the highest glucose concentration were used in the upscale saccharification.

Large-scale Saccharification and Detoxification

Large-scale saccharification based on optimized parameters was done using the saccharification reactor for better handling and mixing thus leading to an efficient saccharification. The resulting slurry was obtained by filtering through several layers of cheese cloth to separate the remaining solids. Then, detoxification of saccharified hydrolysates was performed. Detoxification was done to remove the inhibitory by-products after pre-treatment and saccharification that may hinder ethanol production and also increase the concentration of sugar in the hydrolysate for fermentation. Ion exchange resins, enzymatic detoxification and activated carbon are some of the methods employed to detoxify hydrolysates (Chandel et al., 2011). In our case, activated carbon was used. Activated carbon is known to be a cost-effective method of detoxification that absorbs toxic compounds without affecting the amount of fermentable sugars in the hydrolysate. Detoxification with activated carbon, however, is dependent on many factors such as pH, incubation time, temperature and concentration of activated carbon used (Chandel et al., 2011).

Ethanol Fermentation

Laboratory scale ethanol fermentation (flask fermentation set up) was performed prior to up-scale bioreactor fermentation. Anaerobic fermentation was performed using detoxified and non-detoxified hydrolysates, supplemented with nutrients for alcohol fermentation (AFM). Fermentation performance was observed by measuring the carbon dioxide (CO_2) released in the flask setup, which is directly proportional to the ethanol produced. The CO_2 released was observed to be higher in detoxified hydrolysate than non-detoxified hydrolysate (Fig. 5). During the first 2 h, it was observed that there was a rapid release of CO_2, but then became gradual until the eighth hour. Change in carbon dioxide released was observed to stop after 6 hours of fermentation due to the limited sugar content of the hydrolysate. Maximum growth rate, u_{max}, achieved at the exponential phase was 0.430 hr^{-1} using the non-detoxified hydrolysate, while that for the detoxified hydrolysate was 0.232 hr^{-1}. Apparently, detoxification may have removed some important factors for yeast growth. It was observed that detoxification leads to increased ethanol (in terms of CO_2 formation) production but decreased biomass formation.

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Table 1. Growth and residual sugars formed in various saccharification parameters.

Run No.*	Incubation Time (h)	Solid Loading (w/v)	Enzyme concentration (%)	Growth (Absorbance, 550 nm)	Reducing Sugar (g/L)
1	40	15.00	0.10	0.025	7.00
2	40	15.00	1.00	0.038	24.67
3	20	20.00	0.55	0.027	24.34
4	30	10.00	0.10	0.032	4.00
5	20	15.00	1.00	0.138	13.67
6	30	15.00	0.55	0.012	12.67
7	30	15.00	0.55	0.033	10.67
8	20	15.00	0.10	0.038	9.67
9	20	10.00	0.55	0.073	8.33
10	30	20.00	1.00	0.051	17.00
11	40	20.00	0.55	0.029	12.67
12	40	10.00	0.55	0.041	5.67
13	30	15.00	0.55	0.021	10.67
14	30	15.00	0.55	0.031	11.00
15	30	15.00	0.55	0.074	10.33
16	30	20.00	0.10	0.023	9.00
17	30	10.00	1.00	0.017	7.67

*each flask contains a total volume of 30 mL

For the bioreactor run, the detoxified hydrolysate yielded 2.64 gL^{-1} ethanol, higher than the non-detoxified one (1.49 gL^{-1}). Similarly, detoxification would also result to higher CO_2 production since it is directly proportional to ethanol production. Percent sugar consumption were 60.26% and 65.26% for the detoxified and non-detoxified hydrolysates, respectively. These results are quite less compared with results of previous reports on the ethanol fermentation of saccharified banana pseudo stem (Filho et al., 2013; Souza et al., 2014), perhaps due to less concentration of yeast inoculum used in this study. Further works should focus on increasing the resulting reducing sugar concentration upon saccharification and optimization of the fermentation in order to further increase the ethanol concentration of the final product. Simultaneous saccharification and fermentation using thermotolerant yeast were shown to increase ethanol production in recent studies (Kusmiyati et al., 2017, Islam et al., 2019). Gradual increase of substrate loading upon saccharification, coupled with intermittent cellulase input, could also be a good strategy in future works.

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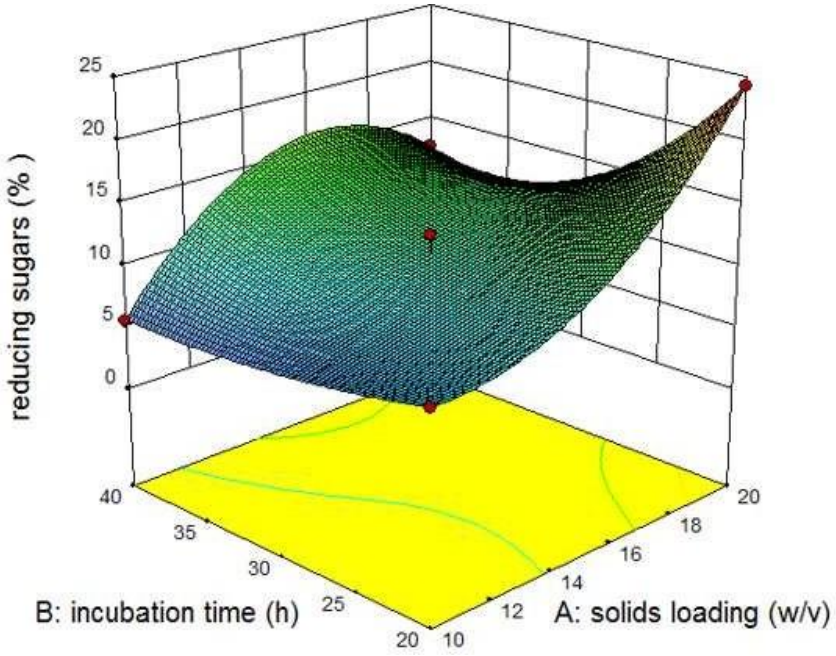

Figure 4. Surface response model graph of Box-Bhenken optimization of enzyme-mediated saccharification at 0.55% enzyme loading

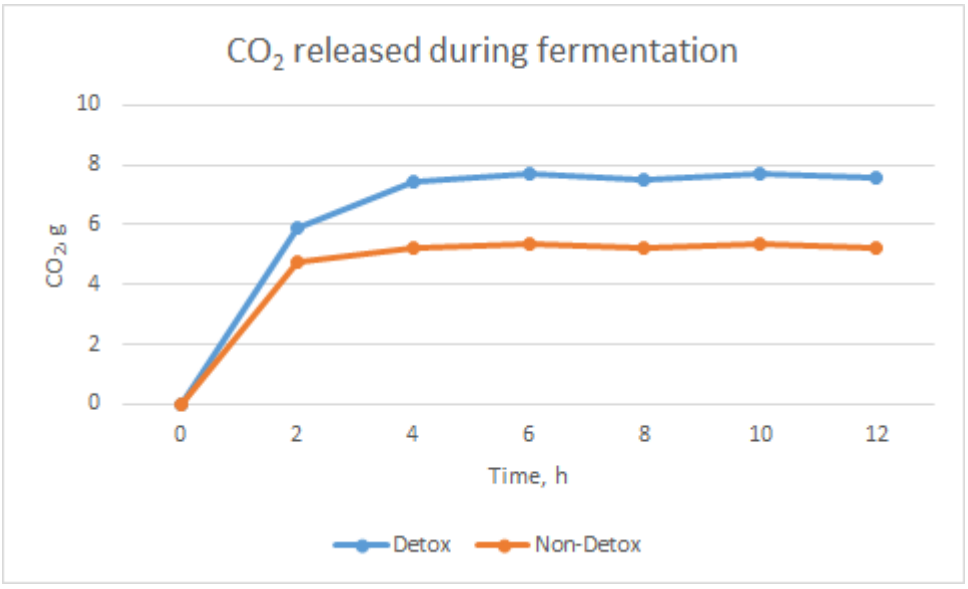

Figure 5. Carbon dioxide released during flask fermentation.

CONCLUSION

The potential use of *M. acuminata x balbisiana* pseudostem as raw material for second generation ethanol production was investigated thru optimization and determination of the effects of different solid loading, incubation time and amount of enzyme on the yield of reducing sugars (YRS). Incubation for 20 h with 15 g solid loading and 0.55% enzyme concentration were the saccharification parameters that resulted to the highest reducing sugar released. Scaled-up saccharification done increased the concentration of sugar in the hydrolysate. Detoxification using activated carbon improved fermentation performance.

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